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Sir:

Transmitted herewith for filing under 37 CFR §1.53(b) is the
[x] patent application of
[ ] continuation patent application of
[ ] divisional patent application of
[ ] continuation-in-part patent application of

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By _____

Inventors: HORST LINDHOFER; HANS-JOACHIM KOLB; REINHARD ZEIDLER; GEORG BORNKAMM

For: METHOD FOR EX VIVO IMMUNIZATION USING HETEROLOGOUS INTACT BISPECIFIC AND/OR TRISPECIFIC ANTIBODIES

[x] This application claims priority from each of the following Application Nos./filing dates: German appl. No. 19725586.8 / June 17, 1997; _____/_____; _____/_____, the disclosure(s) of which is (are) incorporated by reference.

[ ] Please amend this application by adding the following before the first sentence: --This application is a [ ] continuation [ ] continuation-in-part of and claims the benefit of U.S. Provisional Application No. 60/_____, filed _____, the disclosure of which is incorporated by reference.--

Enclosed are:
[x] 6 sheet(s) of [ ] formal [x] informal drawing(s).
[ ] An assignment of the invention to _____.
[x] A [ ] signed [x] unsigned Declaration & Power of Attorney.
[ ] A [ ] signed [ ] unsigned Declaration.
[ ] A Power of Attorney.
[ ] A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27 [ ] is enclosed [ ] was filed in the prior application and small entity status is still proper and desired.
[x] A certified copy of German priority application No. 19725586.8.
[ ] Information Disclosure Statement under 37 CFR 1.97.
[ ] Notification of change of [ ] power of attorney [ ] correspondence address filed in prior application.
[x] Preliminary Amendment

In view of the Unsigned Declaration as filed with this application and pursuant to 37 CFR §1.53(d), Applicant requests deferral of the filing fee until submission of the Missing Parts of Application.

DO NOT CHARGE THE FILING FEE AT THIS TIME.

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80309.fee

Attorney Docket No.: 80309 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of)
)
HORST LINDHOFER, et al.)
)
Serial No: not yet assigned)
)
Filed: herewith)
) PRELIMINARY AMENDMENT
For: METHOD FOR EX VIVO)
IMMUNIZATION USING)
HETEROLOGOUS INTACT)
BISPECIFIC AND/OR TRI-)
SPECIFIC ANTIBODIES)
)

San Francisco, CA 94111
June 15, 1998

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Please make the following amendments to this application.

IN THE CLAIMS:

Claim 9, line 1, please delete "one or more of the preceding claims" and substitute therefor --claim 1--.

Claim 15, line 1, delete "or 14".

Claim 16, line 1, delete "or 14".

Claim 23, line 2, delete "or 14".

Claim 26, line 2, delete "or 14".

REMARKS

Amendment is made to eliminate multiple claim dependencies, thereby avoiding the need to pay the multiple dependent surcharge.

Respectfully submitted,

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Method for ex vivo immunization using heterologous intact bispecific and/or trispecific antibodies

The invention relates to a method for ex vivo immunization using heterologous, intact bispecific and/or trispecific antibodies as well as the use of the products of said method in the prevention and therapy of tumourous diseases and in particular in the induction of an anti-tumour immunity.

Despite the progresses in chemotherapy and radiotherapy achieved during recent years, malignant diseases in humans, for example advanced breast cancer, still have an extraordinarily unfavourable prognosis. Generally, such diseases are impossible to heal. Therefore, it is necessary to develop novel treatment strategies. In this respect, great hopes are placed on immunotherapeutic approaches which shall be used to induce the patient's immune system to reject the tumour. It is well known that tumour-associated antigens are present on tumour cells, and that in principle the immune system is very well able to recognize these antigens and to attack the malignant cells. However, tumours have developed various strategies which enable them to escape the immune reaction. They achieve this for example by an insufficient presentation of

tumour-associated antigens and/or insufficient activation of tumour-specific T cells which are generally present.

With about 43,000 new cases/year, breast cancer occupies a top position in the cancer statistics of women in Germany. Less than one third of the women suffering from lymph node invasion at the time of diagnosis survive for 10 years without relapse.

To date, the immunotherapeutic approaches towards mamma carcinoma have been restricted to methods for unspecific stimulation, such as treatment by BCG or levamisole, and to the use of LAK and NK cells with IL-2 (3, 4). However, the types of immunotherapy employed provided no evidence for a prolongation of life; the treatment by BCG even proved to be disadvantageous (3). Since the unspecific activation of cells has not been very successful also in other types of tumour, attempts were made towards the induction of a specific immune reaction.

For example, T cell-redirecting bispecific antibodies were used in tumour therapy. These antibodies bind with one of their binding arms to a T cell receptor complex and with their other binding arm to a tumour-associated antigen on a tumour cell. The resulting activation of the T cell and the spatial proximity of the tumour cell leads to destruction of the latter by induction of apoptosis or by cytokins, such as TNF-α or perforin, respectively.

The antibodies used in tumour therapy in the prior art were directly infused into patients. This type of procedure shows several disadvantages:

- it requires high doses of antibodies;
- severe side effects may occur;
- by their tumour binding arm the antibodies may also bind to normal tissue during in vivo application.

It is an object of the present invention to provide a novel method for the therapy of malignant diseases in humans, in particular with the objective of achieving an anti-tumour immunity.

According to the invention, this object has been achieved by the method characterized in more detail in claim 1. Preferred embodiments of the method become clear from the dependent claims.

The end product of the method of the present invention is a tumour cell preparation containing antibodies. This tumour cell preparation is used in the prevention and treatment of tumorous diseases by inducing an anti-tumour immunity.

By using the method of the present invention autologous tumour cells are treated with heterologous bispecific and/or trispecific antibodies, and the tumour cell preparation obtained by the present method is used for reinfusion into the patient or the animals from whom the autologous tumour cells have been obtained.

The invention relates further to the use of the method and the tumour cell preparations provided according to the invention in the prevention and therapy of tumourous diseases, in particular in the achievement of an anti-tumour immunity and particularly preferred of a long-term immunity.

The experiments provided in the present invention, particularly example 2, show that a long-lasting tumour immunity is provided. The results of the experiment performed in mice can be transfered also to humans. It is expected that a long-term immunity of several years can be provided by using the present invention. A tumour cell as descibed in the present invention is every cell which has lost its normal func-tion by one or more mutations or wherein its normal function has been changed. Due to these mutations the tumour cells are able to propagate in an uncontrolled manner.

Tumour immunity according to the present invention is defined by activating the immune system of the body in an organism against the autologous tumour in such a way that a long-term or even permanent destruction and/or control of the autologous tumour is achieved.

According to the invention every kind of tumours falling under the definition given above can be treated by the present method. Particularly epithelial tumours, adenocarcinomas, colon carcinomas, mamma carcinomas, overial carcinomas, carcinomas of lungs, throat, nose and ear can be treated. Furthermore, preferably non-epithelial tumours like leukaemias and lymphomas and virus inducted tumours like liver tumours or cervix carcinomas can be treated.

According to the invention, heterologous intact bispecific and/or trispecific antibodies are used. These antibodies are contacted ex vivo with tumour cells (autologous tumour cells) previously obtained from a patient. To prevent the survival of tumour cells following reinfusion, the tumour

cells were treated in a manner known per se, such as by irradiation, prior to contacting with the antibodies. Following irradiation, the tumour cells are incubated with the intact heterologous bispecific and/or trispecific antibodies. According to the invention, not any antibody may be used but only antibodies which are intact, i.e. having a functional Fc portion, and they must be heterologous in nature, i.e. such antibodies which consist of heavy immunoglobulin chains of different subclasses (subclass combinations, also fragments) and/or origin (species).

These intact heterologous bispecific and/or trispecific antibodies will be selected to further have the following properties:

α - binding to a T cell;

β - binding to at least one antigen on a tumour cell;

γ - binding, by their Fc portion (in the case of bispecific antibodies), or by a third specificity (in the case of trispecific antibodies) to Fc receptor-positive cells.

In a particularly preferred embodiment of the present invention the intact heterologous bispecific and/or trispecific antibodies are selected to be able to activate the Fc receptor-positive cell, and thereby inducing or increasing the expression of cytokins and/or co-stimulatory antigens. The tumour cell preparation obtained including said antibodies is them prepared further for reinfusion. It is transferred for instance in a device suitable for reinfusion.

In the case of trispecific antibodies, binding to the Fc

receptor-positive cells preferably takes place via the Fc receptor of Fc receptor-positive cells or also via other antigens on Fc receptor-positive cells (antigen-presenting cells), such as the mannose receptor.

Only the present method and the use of the antibodies described herein ensures the development of an anti-tumour immunity after reinfusion of the antibodies into the patient from whom the tumour cells have previously been obtained. Preferably, reinfusion is carried out in a patient after treatment of the primary tumour, preferably in patients in a minimal residual disease (MRD) situation. In patients with few residual tumour cells but with a high risk of relapse, use of the method provided according to the invention will be particularly successful.

By using the method of the invention, it is possible to avoid the disadvantages known from the prior art and described in more detail above.

The heterologous bispecific and/or trispecific antibodies useful according to the invention are in part known per se, but in part they are described for the first time in the present application. An example for a bsab is antibody anti-CD3 x anti-epcam which is employed in epithelial tumours such as mamma carcinoma.

According to the invention, two variations of the method may be distinguished:

1. short-term incubation, and
2. long-term incubation.

A short-term incubation is an incubation of the autolo-

gous tumour cells with intact heterologous bispecific and/or trispecific antibodies for a period of 10 minutes to 5 hours, or 10 minutes to 3 hours, or further preferred for a period of about 15 minutes to 2 hours, further preferred for a period of 15 minutes to 1 hour. The tumour cells charged with antibodies in this way are then prepared for reinfusion.

The long-term incubation is an incubation of the autologous tumour cells also for a period of about 10 minutes to 5 hours, preferably for a period of 15 minutes to 2 hours and further preferred for a period of 15 minutes to 1 hour, so that the autologous tumour cells are charged with antibodies. Subsequently, blood cells of the patient, preferably mononucleated cells of the peripheral blood (PBMCs = peripheral blood mononucleated cells) are added, and this mixture is then incubated over a prolonged period, such as 1 to 14 days, preferably 3 to 10 days and further preferred 6 to 10 days. Alternatively, another way of proceeding is contacting the autologous tumour cells directly with the bispecific and/or trispecific antibodies and with the patient's blood cells, preferably peripheral blood mononucleated cells. In this way, "priming" of numerous immune cells against the tumour is achieved already ex vivo. Afterwards, these cells are reinfused into the patient. Long-term incubation also leads to internalization and degradation of the antibodies.

Preliminary in vitro results show that immune cells pretreated in the way described are able to destroy tumour cells without further additon of bispecific and/or trispecific antibodies (cf. Example 1).

In short-term as well as long-term incubation, the T cells are redirected to the tumour cells by the bispecific and/or trispecific antibodies which are immobilized on the tumour cells; at the same time binding of Fc receptor-positive cells to the Fc portion of the bispecific and/or trispecific antibody takes place after reinfusion. This leads to activation of Fc receptor-positive cells by their binding to the Fc portions of immobilized (on the T cell or tumour cell, respectively) intact bispecific antibodies.

To enhance the success of immunization, the tumour cells treated with the antibodies either according to the short-term incubation method or the long-term incubation method may be administered to the patient not only once but optionally also several times.

On the tumour cell, an up-regulation of MHC 1 as well as activation of the intracellular processing machinery (proteasome complex) occurs due to the release of cytokins (such as INF-γ and TNF-α) in direct proximity of the tumour cell. Cytokins are released because of the bispecific antibody-mediated activation of T cells and accessory cells (see Figs. 1 and 3). I.e. by the intact bsab not only tumour cells are destroyed and phagocytized but indirectly also their anti-tumour immunity is increased.

Activation of the Fc receptor-positive cells by the bsab depends on the subclass or subclass combination, respectively, of the bsab. As demonstrated in in vitro experiments, for example bsabs of the subclass combination mouse IgG2a/rat IgG2b are able simultaneously to bind to and activate Fc re-

ceptor-positive cells leading to up-regulation and formation (expression), respectively, of co-stimulatory antigens, such as CD40, CD80, or CD86, on the cell surface of such cells. In contrast, bsabs of the subclass combination mouse IgG1/IgG2b are able to bind to Fc receptor-positive cells (1) but clearly are unable to activate these cells to a comparable extend (2).

While the intact bsab at the same time binds to and activates the T cell via one of its binding arms (e.g. to CD3 or CD2), co-stimulatory signals derived from the Fc receptor-positive cell bound to the Fc portion of the bsab may be transferred to the T cell. I.e. only the combination of T cell activation via one binding arm of the bsab and the concomitant transfer of co-stimulatory signals from the Fc receptor-positive cell to the T cell results in an effective T cell activation (Fig. 1A). Tumour-specific T cells which have been insufficiently activated at the tumour cell and are anergic may also be reactivated according to the ex vivo pretreatment of the invention (Fig. 1B).

A further important aspect in the induction of anti-tumour immunity is the possibility of phagocytosis, processing and presentation of tumour components by accessory cells (monocytes/macrophages, dendritic cells, and NK - "natural killer" - cells) which have been directed and activated by the bsab. By this classical mechanism of antigen presentation tumour-specific CD4 cells as well as CD8 positive cells can be generated. Moreover, tumour-specific CD4 cells play an important role in the induction of a humoral immune reaction

in the context of the T-B cell cooperation.

Bispecific and trispecific antibodies are able to bind to the T cell receptor complex of the T cell by one binding arm and to tumour-associated antigens on the tumour cells by the second binding arm. Thereby, they activate T cells which destroy the tumour cells by releasing cytokins or apoptosis-mediating mechanisms. Furthermore, in the context of their activation by bispecific antibodies it is clearly possible for T cells to recognize tumour-specific antigens via their receptor whereby a long-lasting immunization is initiated (Fig. 1B). In this respect, the intact Fc portion of the bi-specific or trispecific antibodiy is of particular importance mediating the binding to accessory cells such as monocytes/-macrophages and dendritic cells and inducing these cells to become themselves cytotoxic and/or simultaneously transfer important co-stimulatory signals to the T cell (Fig. 1B). In this manner, it seems to be possible that a T cell reaction may be induced also against so far unknown tumour-specific peptides.

Redirection of possibly anergized tumour-specific T cells to tumour cells by means of bispecific and/or trispeci-fic antibodies and concomitant co-stimulation of such T cells by accessory cells bound to the Fc portion of the bispecific or trispecific antibody might act to reverse the anergy of cytotoxic T cells (CTLs). I.e. using intact heterologous bi-specific and/or trispecific antibodies a T cell tolerance existing in the patient against the tumour may be neutralized and, thereby, a long-lasting anti-tumour immunity may be in-

duced.

The last aspect is supported by preliminary in vivo data from experiments with mice indicating a long-lasting anti-tumour immunity following treatment with a syngeneic tumour and intact bsab. In these experiments a total of 14 out of 14 animals which could be successfully treated with bsab after a first tumour injection survived another tumour injection 144 days after the first one - without further administration of bsab (see Example 2).

Further advantages in the ex vivo immunization by bispecific and/or trispecific antibodies are (i) minimizing possible side effects, (ii) controlled binding of the tumour binding arm to the tumour cells outside of the body, and (iii) use of as little bispecific and trispecific antibodies as possible. Principally, there are two different ways of proceeding which will be detailed in the following. An important aspect with long-term incubation is that the bispecific or trispecific antibody employed is exhausted and degraded during the incubation period planned. In this way, this immunization would avoid the lengthy drug approval process.

In the short-term and long-term incubation procedures, the tumour cells are incubated with antibodies over a period of 10 minutes to 5 hours, preferably up to 3 hours, further preferred up to 2 hours and still further preferred 15 minutes to 1 hour. Preferably, the incubation is carried out at a temperature of 4°C to 25°C, particularly preferred 4°C to 10°C. The incubation is preferably performed in a sterile

environment in bufferd saline having a neutral pH. In the case of short-term incubation, reinfusion into the patient is performed immediately afterwards. In the long-term incubation procedure, following this preincubation mononucleated peripheral blood cells are added and incubated together with the preincubated tumour cells/antibodies for a further period of 1 to 14 days, more preferably 3 to 10 days, further preferred 6 to 10 days. Preferably, this incubation is performed at 37°C under sterile conditions as well as under GMP conditions (Good Manufacturing Production = GMP) in an incubator. As detailed above, in long-term incubation the blood cells may alternatively be incubated together with tumour cells and antibodies under suitable conditions.

The incubation conditions described above are only intended to be an example. Depending on the tumour cells and the antibodies used also other time periods, temperature conditions etc., and in general different incubation conditions may be used. By simple experimentation, the skilled artisan will be able to establish such conditions.

During preincubation the tumour cells are preferably employed in an amount of 10^7 to 10^9 cells, further preferred in an amount of about 10^8 cells. The peripheral blood mononucleated cells are added in an amount of about 10^8 to 10^{10} cells. Naturally, the skilled artisan may select different incubation conditions which may be determined by laboratory experimentation (for example changes in cell number and incubation period). The bi-specific and/or tri-specific antibodies used in the method of the present invention are added in

an amount of 2 to 100 µg, more preferably 5 to 70 µg, particularly preferred 5 to 50 µg.

The autologous tumour cells employed are for example irradiated to prevent further survival of tumour cells. For example, gamma radiation is used e.g. employed in a radiation dose of 50 to 100 Gy. In another embodiment of the present invention the autologous tumour cells are treated by chemical substances, for instance by mitomycin C to prevent their further survival.

The antibodies used according to the invention are preferably able to reactivate tumour-specific T cells being in an anergic state. Further, they are able to induce tumour-reactive complement-binding antibodies and thereby a humoral immune reaction.

Binding preferably takes place via CD3, CD2, CD4, CD5, CD6, CD8, CD28, and/or CD44 to the T cell. Fc receptor-positive cells at least bear a Fcγ receptor I, II, or III.

Antibodies which may be employed according to the invention are able to bind to monocytes, macrophages, dendritic cells, "natural killer" cells (NK cells) and/or activated neutrophils being Fcγ receptor 1-positive cells.

The antibodies which may be used according to the invention lead to an induction or increase in the expression of CD40, CD80, CD86, ICAM-1, and/or LFA-3 as co-stimulatory antigens and/or cytokin secretion by the Fc receptor-positive cell. The cytokins preferably are IL-1, IL-2, IL-4, IL-6, IL-8, IL-12, and/or TNF-α.

Binding to the T cell preferably takes place via the T

cell receptor complex of the T cell.

The bispecific antibodies which may be used according to the invention preferably are:

- an anti-CD3 X anti-tumour-associated antigen antibody and/or anti-CD4 X anti-tumour-associated antigen antibody and/or anti-CD5 X anti-tumour-associated antigen antibody and/or anti-CD6 X anti-tumour-associated antigen antibody and/or anti-CD8 X anti-tumour-associated antigen antibody and/or anti-CD2 X anti-tumour-associated antigen antibody and/or anti-CD28 X anti-tumour-associated antigen antibody and/or anti-CD44 X anti-tumour-associated antigen antibody.

The trispecific antibodies which may be employed according to the invention preferably are:

- an anti-CD3 X anti-tumour-associated antigen antibody and/or anti-CD4 X anti-tumour-associated antigen antibody and/or anti-CD5 X anti-tumour-associated antigen antibody and/or anti-CD6 X anti-tumour-associated antigen antibody and/or anti-CD8 X anti-tumour-associated antigen antibody and/or anti-CD2 X anti-tumour-associated antigen antibody and/or anti-CD28 X anti-tumour-associated antigen antibody and/or anti-CD44 X anti-tumour-associated antigen antibody.

The trispecific antibodies useful according to the invention at least have a T cell binding arm, a tumour cell binding arm and a binding arm which binds to Fc receptor positive cells. The latter of the binding arms mentioned may be an anti-Fc receptor binding arm or a mannose receptor binding arm.

The bispecific antibody preferably is a heterologous

intact rat/mouse bispecific antibody.

By the bispecific and trispecific antibodies useful according to the invention T cells are activated and redirected against the tumour cells. Heterologous intact bispecific antibodies which may be preferably used are selected from one or more of the following isotype combinations:

rat-IgG2b/mouse-IgG2a,

rat-IgG2b/mouse-IgG2b,

rat-IgG2b/mouse-IgG3;

rat-IgG2b/human-IgG1,

rat-IgG2b/human-IgG2,

rat-IgG2b/human-IgG3[oriental allotype G3m(st) = binding to protein A],

rat-IgG2b/human-IgG4;

rat-IgG2b/rat-IgG2c;

mouse-IgG2a/human-IgG3[caucasian allotypes G3m(b+g) = no binding to protein A, in the following indicated as *]

mouse-IgG2a/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-IgG2a/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-IgG2a/human-[VH-CH1,VL-CL]-human-IgG1-[hinge]-hu-

man-IgG3*-[CH2-CH3]

mouse-[VH-CH1,VL-CL]-human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-[VH-CH1,VL-CL]-human-IgG4/rat-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG4[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2: > aa position 251]-human-IgG3*[CH3]

rat-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge-CH2-CH3]

rat-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG2-[hinge-CH2-CH3]

rat-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG3-[hinge-CH2-CH3, oriental allotype]

rat-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG4-[hinge-CH2-CH3]

human-IgG1/human-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG4[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

human-IgG1/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG4[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]
human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG2[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

human-IgG1/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG2[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG1/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG2/human-[VH-CH1,VL-CL]-human-IgG2-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG4/human-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG4/human-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG4[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

mouse-IgG2b/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-IgG2b/human-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-[VH-CH1,VL-CL]-human-IgG4/rat-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG4-[CH2]-human-IgG3*-[CH3]

human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG4-[CH2]-human-IgG3*-[CH3]

human-IgG1/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG4-[CH2]-human-IgG3*-[CH3]

human-IgG4/human-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG4-[CH2]-human-IgG3*-[CH3]

The antibodies useful according to the invention preferably are monoclonal, chimeric, recombinant, synthetic, semi-synthetic or chemically modified intact antibodies having for example Fv, Fab, scFv or $F(ab)_2$ fragments.

Preferably used are antibodies or derivatives or fragments of human origin or antibodies altered in a way that makes them suitable for application to humans (so-called "hu-

manized antibodies") (see for example Shalaby et al., J. Exp. Med. 175 (1992), 217; Mocikat et al., Transplantation 57 (1994), 405).

The preparation of the various types of antibodies and fragments mentioned above is obvious to one skilled in the art. The preparation of monoclonal antibodies preferably originating from mammals, e.g. humans, rat, mouse, rabbit or goat, may be performed using conventional methods, as for example described in Köhler and Milstein (Nature 256 (1975), 495), in Harlow and Lane (Antibodies, A Laboratory Manual (1988), Cold Spring Harbour) or in Galfré (Meth. Enzymol. 73 (1981), 3).

Furthermore, it is possible to prepare the antibodies described by means of recombinant DNA technology according to techniques obvious to the skilled artisan (see Kurucz et al., J. Immunol. 154 (1995), 4576; Hollinger et al., Proc. Natl. Acad. Sc. USA 90 (1993), 6444).

The antibodies used in the present method can be designed and manufactured by a person skilled in the art without undue burden. The enclosed list of references, particularly references (7) to (11) describe methods on how to obtain bispecific and trispecific antibodies to be used in the present invention.

Particularly document (9) of Greenwood et al. discloses the exchange of single immunoglobulin domains (for instance CH2) by suitable cloning technique. By using these cloning technique novel antibody combinations described for instance in claim 9 can be provided. Examples are:

human-(VH-CH1, VL-CL)-human IgG4-(hinge)-human IgG4 (N-terminale region of CH2)-human IgG3* (C-terminal region of CH2:> aminoacid position 251)-human IgG3* (CH3).

The combination with an antibody: human IgG4 for the preparation of the bispecific antibody: human IgG4/human-(VH-CH1, VL-CL)-human IgG4-(hinge)-human IgG4 (N-terminal region of CH2)-human IgG3* (C-terminal region of CH2:>aminoacid position 251)-human IgG3* (CH3) is prepared by simple cell fusion as described for instance in document (6).

On the one hand, the preparation of antibodies having two different specificities, the so-called bispecific antibodies, may be performed using recombinant DNA technologie, but on the other hand also by the so-called hybrid-hydridoma fusion technique (see for example Milstein et al., Nature 305 (1983), 537). By this technique, hybridoma cell lines producing antibodies each having one of the desired specificities are fused, and recombinant cell lines producing antibodies with both specificities are identified and isolated.

The problem underlying the invention may be solved both by bispecific and by trispecific antibodies insofar as they show the features and activities characterized in claim 1. In the following, the preparation of antibodies having two and three specificities is described in more detail. To provide such bispecific and trispecific antibodies belongs to the state of the art, and the literature describing such methods of preparation is hereby incorporated by reference in its entirety.

The preparation of antibodies having three specifici-

ties, so-called trispecific antibodies, which are also suitable to solve the fundamental problem of the invention may be for example carried out by coupling to one of the heavy IgG chains of a bispecific antibody a third antigen-binding site having another specificity, e.g. in the form of "single chain variable fragments" (scFv). The scFv may be for example bound to one of the heavy chains via a

$$-S\text{-}S(G_4S)_nD\text{-}I \text{ linker}$$

(S = serine, G = glycine, D = aspartate, I = isoleucine).

Analogously, trispecific $F(ab)_2$ constructs may be prepared substituting the CH2-CH3 regions of the heavy chain of one specificity of a bispecific antibody by a scFv of a third specificity while the CH2-CH3 regions of the heavy chain of the other specificity are removed, e.g. by introduction of a stop codon (at the end of the "hinge" region) into the coding gene for example by homologous recombination (see Fig. 5).

It is also possible to prepare trispecific scFv constructs. In this case three VH-VL regions representing three different specificities are arranged in series (Fig. 6).

According to the invention, there are for example used intact bispecific antibodies. Intact bispecific antibodies are a combination of two antibody semi-molecules (each of one H and L immunoglobulin chain) each representing one specificity and, like normal antibodies, having in addition a Fc portion which performs the well known effector functions. Preferably, they are prepared by quadroma technology. This method of preparation is described representatively in DE-A-44 19 399. This document is incorporated by reference in its

entirety for the purpose of complete diclosure also with respect to a definition of bispecific antibodies. Naturally, also other methods of preparation may be employed as long as they result in the intact bispecific antibodies defined above required according to the invention.

For example, by a newly developed method of production (6) intact bispecific antibodies may be prepared in a sufficient amount. The combination of 2 bispecific antibodies against 2 different tumour-associated antigens (e.g. c-erb-B2, ep-cam, such as GA-733-2 = C215) on the mamma carcinoma cells minimizes the risk that tumour cells expressing only one antigen are not recognized.

There have also been attempts to achieve an anti-tumour immunity by treatment with bispecific F(ab´)2 fragments having the specificities of anti-c-erb-B2 x anti CD64. The main disadvantage of bsF(ab´)2 fragments is that due to the specificities used only FcγRI+ cells are redirected to the tumour. T cells are not redirected to the tumour by this bispecific antibody. While bsF(ab´)2 fragments have the potenntial to directly destroy the tumour, they are unable to establish an anti-tumour immunity themselves. Only the T cell with its specific T cell receptor has this capability. While the FcγRI+ cells are able to indirectly activate tumour-specific T cells by presenting tumour-specific peptides (via MHCI or MHCII, respectively), for example following phagocytosis of tumour components, the efficiency of induction of an anti-tumour immunity is this case is not as high (only in 30% of the patients).

Further advantages of intact bsabs capable of redirecting T cells as compared to the above-mentioned bsF(ab´)2 fragments are detailed in the following:

1. To intact bsabs there may bind Fc receptor-positive cells and may on the one hand by ADCC (antibody-dependent cell-mediated cytotoxicity) contribute directly to the destruction of the tumour and on the other hand to T cell activation, as detailed above.

2. By intact T cell-redirecting bsabs also anergized tumour-specific T cells are directed to the tumour cell which according to the invention may be directly reactivated at the tumour. This may not be achieved using a bsF(ab´)2 fragment having the specificities of anti CD64 x anti tumour-associated antigen.

3. A bsF(ab´)2 fragment having the specificities of anti CD64 x anti tumour-associated antigen is merely able of achieving an anti-tumour immunity in 30% of the patients while according to the invention in experiments with mice using T cell-redirecting intact bsabs a protection in 100% of the animals could be achieved.

Binding of the bsabs to Fcγ-RI has two significant advantages with respect to optimum anti-tumour effectivity:

(1) Fcγ-RI-positive cells are capable of eliminating tumour

cells by means of ADCC (11) and in this respect may contribute synergistically to the anti-tumour-effect of the cytotoxic T cells which have been directed to the tumour cell by the bsab (13).

(2) Fcγ-RI-positive cells (such as monocytes/macrophages/dendrites) are capable of providing important co-stimulatory signals similar to antigen presentation to the T cell and thereby to prevent anergizing of the T cell. Furthermore, as shown in Figure 1, as a desired side product due to the intact bsab-mediated interaction of the T cell with accessory cell and tumour cell there may be stimulated T cells having a T cell receptor which recognizes tumour-specific peptides (presented on the tumour cell via MHC antigens). The co-stimuli necessary for a correct activation of the T cell in this situation would be provided by the accessory cell (e.g. the monocyte). In this respect, the antibody of the invention besides the direct T cell receptor-independent bsab-mediated tumour destruction (Fig. 1A) should also activate and generate tumour-specific T cells (Fig. 1B) which after degradation of the bsabs continue to patrol in the patient. I.e. by means of intact bsabs similar to genetherapeutical approaches (e.g. by incorporation of co-stimulatory antigens such as B-7 into the tumour cell) the tumour tolerance in the patient may be overcome.

In this respect, it is further beneficial that the expression of Fcγ-RI is up-regulated on the respective cells following G-CSF treatment.

The invention has been described in the above and will be described in the following in particular with respect to bispecific antibodies. Instead of bispecific antibody, of course also trispecific antibodies may be used as long as they comply with the provisions made.

The invention has been and will be described with respect to the accompanying Figures. The Figures show:

Fig. 1: the role of accessory cells in tumour immunotherapy by means of bispecific antibodies;

Fig. 2: the destruction of tumour cells following administration of bispecific antibodies as evidenced by flow-cytometry;

Fig. 3: induction of cytokins by intact bispecific antibodies only but not by parental antibodies;

Fig. 4: efficiency of the method according to the invention in vivo;

Fig. 5: trispecific $F(ab)_2$ antibodies;

Fig. 6: trispecific scFv antibody.

IMMUNIZATION PROTOCOLS

Ex vivo immunization (short-term incubation)

1. Preparation of a single cell suspension (10^7-10^9 cells) from autologous tumour material (or allogenic tumour cells of the same tumour type) with subsequent γ irradiation (50-100 Gy).
2. Addition of bsabs (5-50 μg) and incubation for 45 minu-

tes at 4°C. Afterwards washing away of unbound antibodies.

3. Reinfusion of the cell mixture (i.v.).

Ex vivo immunization (long-term incubation)

1. Preparation of a single cell suspension (10^7-10^9 cells) from autologous tumour material (or allogenic tumour cells of the same tumour type) with subsequent γ irradiation (50-100 Gy).
2. Addition of bsabs (5-50 µg), 45 minutes incubation.
3. Addition of PBMCs (10^8-10^{10}), [alternatively: 1 x 10^9 cells obtained from T cell aphaeresis].
4. After 5 to 7 days monitoring of T cell reactivity by transfer of aliquots e.g. to allogenic breast cancer cell lines (MCF-7, MX-1).
5. Reinfusion (i.v.) of the cultured PBMCs on days 4 to 14 into the patient (in the case of T cell aphaeresis: cryo conservation).

Abbreviations: PBMCs, peripheral blood mononucleated cells; i.v., intravenously.

A similar assay but instead depending on the addition of cytokins and carried out using conventional bsabs (no activation of accessory cells by bsabs of the subclass combination rat IgG2B x rat IgG1) demonstrates the principal effectivity of such an ex vivo immunization in the animal model (5).

In contrast to this, the advantage of the method disclosed herein resides in the "self-sufficiency" with respect to cytokins (such as INF-α or TNF-α) required for an up-regulation of for example MHC 1 on the tumour cell by simultaneous activation of T cells and accessory cells (monocytes/macrophages, Fig.) on the tumour cell. This is achieved by the particular subclass combination mentioned at the beginning of the intact bsab used herein. In the case of short-term incubation these processes take place in the patient. Further advantages in short-term incubation are (i) avoiding the cultivation of the cell suspension with serum-containing medium otherwise necessary. (ii) Due to this, also the cost-intensive cultivation according to GMP regulations may be ommitted. (iii) A further important aspect is avoidance or reduction, respectively, of possible side effects by the bsab because of the significantly lower amount of antibodies applied.

An advantage in long-term incubation is that the bsab in vitro after some time exhausts itself (and, thus, this method may be established not as a medicament but as a "medical device").

EXAMPLE 1

Bispecific antibody-mediated lysis of tumour cells by allogenic T cells

H-Lac78 is a cell line which has been established from a hypopharynx carcinoma and which expresses epcam to a high extent (own FACS data). Using H-Lac78 and peripheral mononucleated cells (PBMC) from volunteers it was possible to de-

tect the generation of allogenic cytotoxic T lymphocytes. For this purpose, constant amounts of H-Lac78 (2×10^4) were incubated with varying amounts of PBMCs in the presence (10 ng) or absence of a bsab (anti epcam x anti CD3). After a period of seven days the PBMCs were removed and analysed in a flow-cytometer. At the same time, the number of H-Lac78 tumour cells was determined. The activation of T cells may be observed microscopically by means of cluster formation; proliferation may be evidenced by the incorporation of radiolabeled thymidine. The detection of remaining tumour cells is performed microscopically as well as by the epithelial marker epcam which is not expressed on peripheral blood cells. As shown in Fig. 2, the H-Lac78 cells were completely lysed in the presence of bsab, i.e. no epcam-positive cells were detectable in the flow-cytometer after seven days. These data were confirmed by microscopic oberservations. In contrast, without bsab a confluent layer of H-Lac78 cells was observed in the wells and epcam-positive cells were detectable by FACS.

Detection of activated allospecific CTLs by transfer experiment

In a subsequent transfer experiment the PBMCs incubated with or without bsab, respectively, were transferred onto new H-Lac78 cells without readdition of bsab. Also in this case, the tumour cells were lysed but exclusively by PBMCs which had been activated by bsab previously. H-Lac78 lysis was complete within 24 hours up to a ratio of 2 PBMCs to 1 H-Lac78 cell. This result indicates the generation of allospecific

CTLs without external addition of interleukin-2 (IL-2). Since IL-2 is essential for the activation of T lymphocytes, the data obtained herein suggest that by bsab-mediated activation IL-2 is produced by the T cells themselves. Induction of IL-2 mRNA by addition of bsab could be confirmed afterwards by RT-PCR where the bsab was clearly superior to the parental starting antibodies (Fig. 3). This observation is important insofar as IL-2 has been described as an anti-tumour effective cytokin; but the systemic administration of which in an appropriate concentration is limited because of its toxicity. In contrast, the risk of toxicity does not appear in the local production of IL-2 as it is for example induced by intact bsab. Also, since an effective induction of IL-2 (and IL-12) requires stimulation of T cells via the T cell receptor and CD28, this indicates the importance of Fc receptor-positive cells (providing the ligands for CD28, CD80, and CD86) in T cell activation by intact bsab.

BEISPIEL 2

To address the question whether bispecific antibodies are able to induce a long-lasting anti-tumour immunity C57BL/6 mice were first injected with 5 x 10^3 syngeneic B16 tumour cells. Two days later, a group of mice (number of 18) were treated with intact bsab prepared by quadroma technology (6) and recognizing a target structure (ep-cam/C215 = tumour-associated antigen) on the tumour cell as well as CD3 on the T cells. A second group (number of 6) received an equimolar amount of Fab fragments of both of the specificities contai-

ned in the bsab only. While all of the animals of the Fab control group died or had to be sacrificed within 56 days, 14 of the 18 animals treated with bsab survived. 144 days after the first injection of tumour cells the 14 surviving animals were injected with another dose of 750 B16 tumour cells but this time without administration of bsabs. As a control, the same number of tumour cells was administered to 5 untreated animals. While the last animal of the untreated control group had to be sacrificed 66 days after tumour injection, all of the animals treated with bsab survived (monitoring period: 120 days following second tumour cell injection). See also Figures 4A and B: Survival graphs of the two subsequent experiments described above.

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Method for ex vivo immunization using heterologous intact bispecific and/or trispecific antibodies

C L A I M S

1. Method for ex vivo immunization of humans and animals comprising the following steps of:

a) isolating autologous tumour cells;

b) treating the tumour cells to prevent the survival thereof following reinfusion;

c) incubating the thus treated tumour cells with intact heterologous bispecific and/or trispecific antibodies showing the following properties:

α - binding to a T cell;

β - binding to at least one antigen on a tumour cell;

γ - binding, by their Fc portion (in the case of bispecific antibodies), or by a third specificity (in the case of trispecific antibodies) to Fc receptor-positive cells.

2. Method according to claim 1,
characterized in that
said antibodies are selected so that they are capable of binding Fc receptor-positive cells having a Fcγ receptor I, II, or III.

3. Method according to claim 2,
characterized in that
said antibodies are capable of binding to monocytes, macrophages, dendritic cells, "natural killer" cells (NK cells) and/or activated neutrophils being Fcγ receptor I-positive cells.

4. Method according to claim 1,
characterized in that
said antibodies are capable of inducing tumour-reactive complement-binding antibodies and thus inducing a humoral immune response.

5. Method according to claim 1,
characterized in that
said antibodies are selected to bind to the T cells via CD2, CD3, CD4, CD5, CD6, CD8, CD28 and/or CD44.

6. Method according to claim 1,
characterized in that
said antibodies are selected so that following their

binding to the Fc receptor-positive cells the expression of CD40, CD80, CD86, ICAM-1 and/or LFA-3 as co-stimulatory antigens, and/or secretion of cytokins by the Fc receptor-positive cell is initiated or increased.

7. Method according to claim 6,
characterized in that
said antibodies are selected so that the secretion of IL-1, IL-2, IL-4, IL-6, IL-8, IL-12 being cytokins and/or of TNF-α is increased.

8. Method according to claim 1,
characterized in that
said bispecific antibody is selected to be an anti-CD3 X anti-tumour-associated antigen antibody and/or anti-CD4 X anti-tumour-associated antigen antibody and/or anti-CD5 X anti-tumour-associated antigen antibody and/or anti-CD6 X anti-tumour-associated antigen antibody and/or anti-CD8 X anti-tumour-associated antigen antibody and/or anti-CD2 X anti-tumour-associated antigen antibody and/or anti-CD28 X anti-tumour-associated antigen antibody and/or anti-CD44 X anti-tumour-associated antigen antibody.

9. Method according to one or more of the preceding claims,
characterized in that
said bispecific antibody is selected from one or more of

the following isotype combinations:

rat-IgG2b/mouse-IgG2a,

rat-IgG2b/mouse-IgG2b,

rat-IgG2b/mouse-IgG3;

rat-IgG2b/human-IgG1,

rat-IgG2b/human-IgG2,

rat-IgG2b/human-IgG3[oriental allotype G3m(st) = binding to protein A],

rat-IgG2b/human-IgG4;

rat-IgG2b/rat-IgG2c;

mouse-IgG2a/human-IgG3[caucasian allotypes G3m(b+g) = no binding to protein A, in the following indicated as *]

mouse-IgG2a/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-IgG2a/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-IgG2a/human-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-[VH-CH1,VL-CL]-human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-[VH-CH1,VL-CL]-human-IgG4/rat-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG4[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2: > aa position 251]-human-IgG3*[CH3]

rat-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge-CH2-CH3]

rat-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG2-[hinge-CH2-CH3]

rat-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG3-[hinge-CH2-CH3, oriental allotype]

rat-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG4-[hinge-CH2-CH3]

human-IgG1/human-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG4[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

human-IgG1/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG4[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG2[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

human-IgG1/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG2[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG1/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG2/human-[VH-CH1,VL-CL]-human-IgG2-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG4/human-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG3*-[CH2-CH3]

human-IgG4/human-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG4[N-terminal region of CH2]-human-IgG3*[C-terminal region of CH2 : > aa position 251]-human-IgG3*[CH3]

mouse-IgG2b/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-IgG2b/human-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-IgG2b/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG3*-[CH2-CH3]

mouse-[VH-CH1,VL-CL]-human-IgG4/rat-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG4-[CH2]-human-IgG3*-[CH3]

human-IgG1/rat-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG4-[CH2]-human-IgG3*-[CH3]

human-IgG1/mouse-[VH-CH1,VL-CL]-human-IgG1-[hinge]-human-IgG4-[CH2]-human-IgG3*-[CH3]

human-IgG4/human-[VH-CH1,VL-CL]-human-IgG4-[hinge]-human-IgG4-[CH2]-human-IgG3*-[CH3]

10. Method according to claim 1,
characterized in that
said bispecific antibody is selected from a heterologous rat/mouse bispecific antibody.

11. Method according to claim 1,
characterized in that
said trispecific antibody has a T cell binding arm, a tumour cell binding arm and a third specificity for binding to Fc receptor-positive cells.

12. Method according to claim 11,
characterized in that
said trispecific antibody is selected to be an anti-CD3 X anti-tumour-associated antigen antibody and/or anti-CD4 X anti-tumour-associated antigen antibody and/or anti-CD5 X anti-tumour-associated antigen antibody and/or anti-CD6 X anti-tumour-associated antigen antibody and/or anti-CD8 X anti-tumour-associated antigen antibody and/or anti-CD2 X anti-tumour-associated antigen antibody and/or anti-CD28 X anti-tumour-associated antigen antibody and/or anti-CD44 X anti-tumour-associated antigen antibody.

13. Method according to claim 1,
characterized in that
in said step c) after incubating the tumour cells with intact heterologous bispecific and/or trispecific antibodies the tumour cells charged with antibodies are prepared for reinfusion (short-term incubation).

14. Method according to claim 1,
characterized in that
in said step c) the incubation of the tumour cells with antibodies is performed together with mononucleated cells of the peripheral blood (PBMC = peripheral blood mononucleated cells), or mononucleated cells are added after incubation of the tumour cells with the antibodies

and the incubation is continued (long-term incubation).

15. Method according to claim 13 or 14,
characterized in that
said tumour cells are incubated with the antibodies for a period of 10 minutes to 5 hours.

16. Method according to claim 13 or 14,
characterized in that
said tumour cells are incubated with the antibodies for a period of 15 minutes to 120 minutes.

17. Method according to claim 14,
characterized in that
said mononucleated peripheral blood cells are incubated with the tumour cells and the antibodies for a period of 1 to 14 days.

18. Method according to claim 14,
characterized in that
said mononucleated peripheral blood cells are added in an amount of about 10^8 to 10^{10} cells.

19. Method according to claim 1,
characterized in that
said tumour cells are added in an amount of 10^7 to 10^9 cells.

20. Method according to claim 1,
characterized in that
said bispecific and/or trispecific antibodies are added in an amount of 2 to 100 μg.

21. Method according to claim 1,
characterized in that
said treating of the tumour cells in step b is performed by irradiation.

22. Method according to claim 1,
characterized in that,
said bispecific and/or trispecific antibodies are capable of activating the Fc receptor-positive cell whereby the expression of cytokins and/or co-stimulatory antigens is induced or increased.

23. Use of the tumour cell containing preparation according to claim 1 or 14 in the prevention and treatment of tumourous diseases.

24. Use according to claim 23 for inducing an anti-tumour immunity.

25. Method according to claim 1 for the preparation of autologous tumour cells treated with heterologous bispecfic and/or trispecific antibodies for reinfusion
into the patient or the animals from whom the autologous

tumour cells have been obtained.

26. A pharmaceutical composition containing a tumour cell preparation obtained by the method of claim 1 or 14.

SUMMARY

According to the invention there is described a method for ex vivo immunization of humans and animals comprising the following steps of:

a) isolating autologous tumour cells;

b) treating the tumour cells to prevent the survival thereof following reinfusion;

c) incubating the thus treated tumour cells with intact heterologous bispecific and/or trisepcific antibodies showing the following properties:

α - binding to a T cell;

ß - binding to at least one antigen on a tumour cell;

γ - binding, by their Fc portion (in the case of bispecific antibodies), or by a third specificity (in the case of trispecific antibodies) to Fc receptor-positive cells.

Fig. 1:
The role of accessory cells in tumour immunotherapy
by means of bispecific antibodies

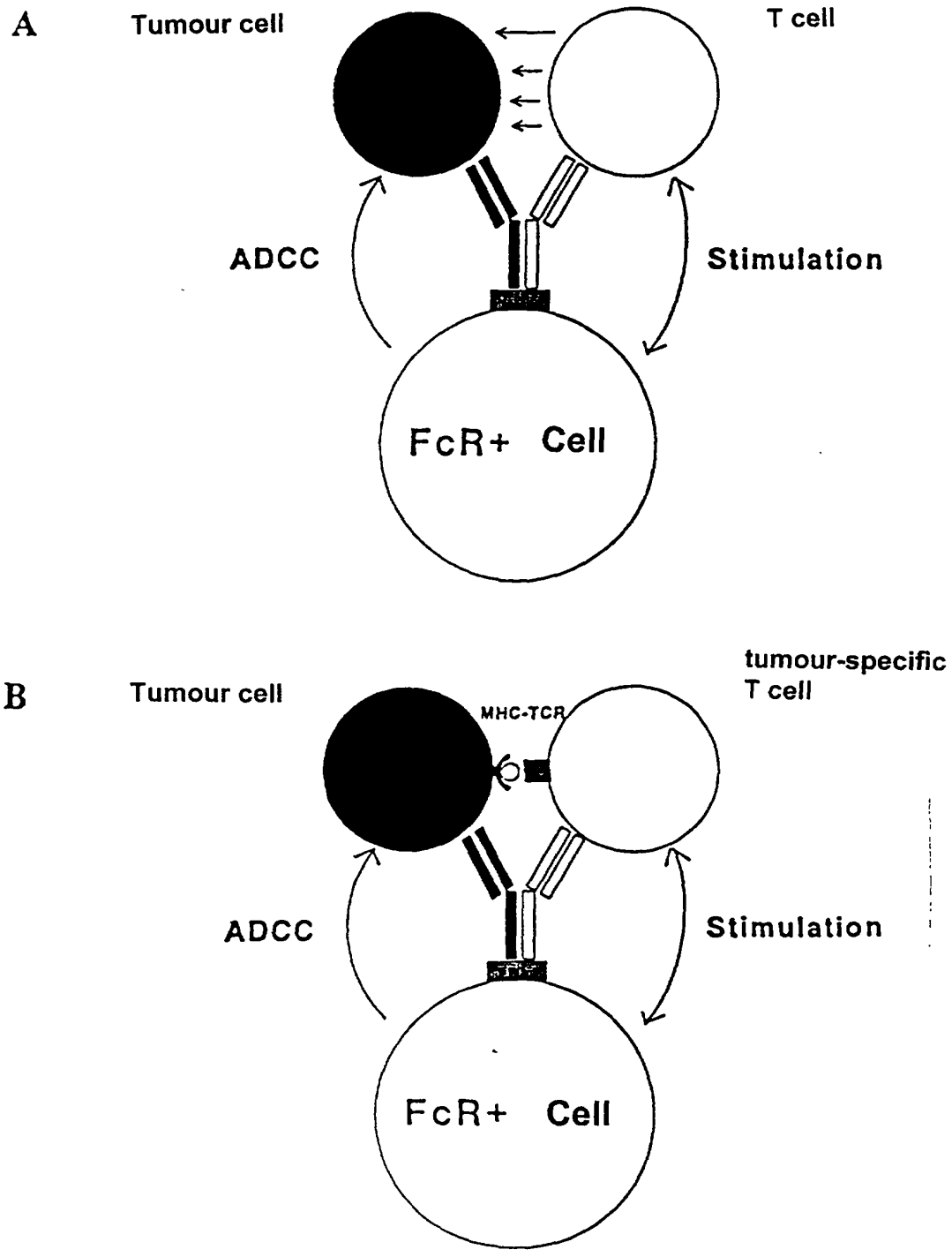


Figure 2

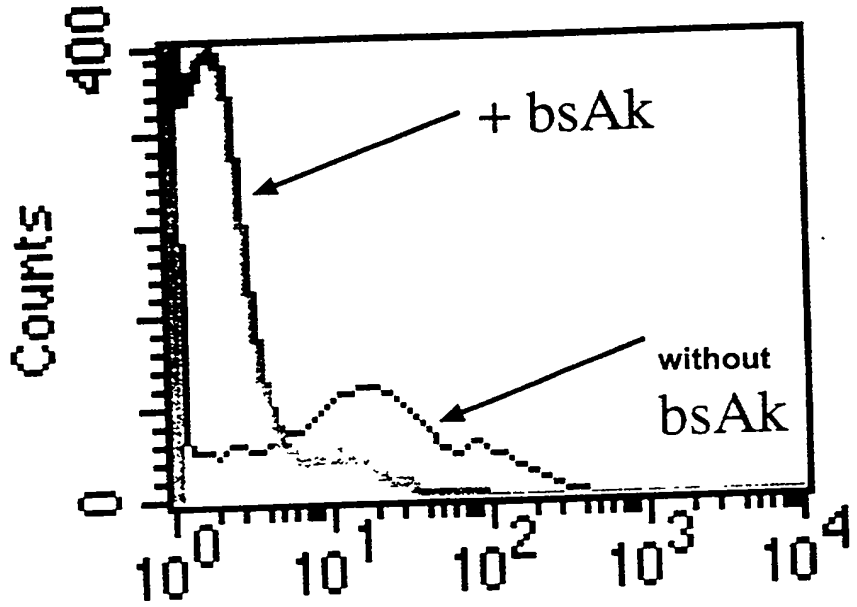


Figure 3

Induction of cytokins by intact bsabs
but not by parental abs

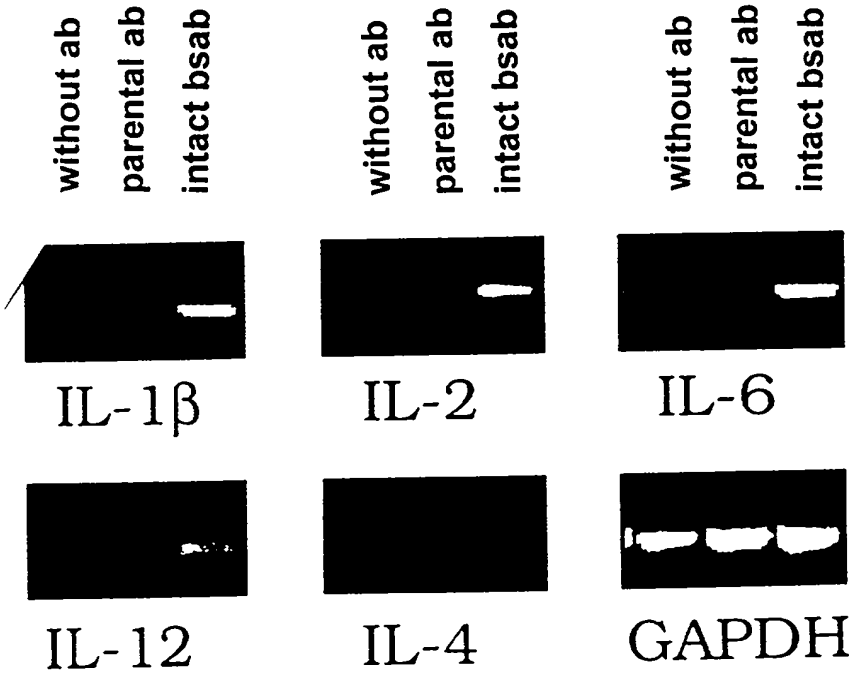
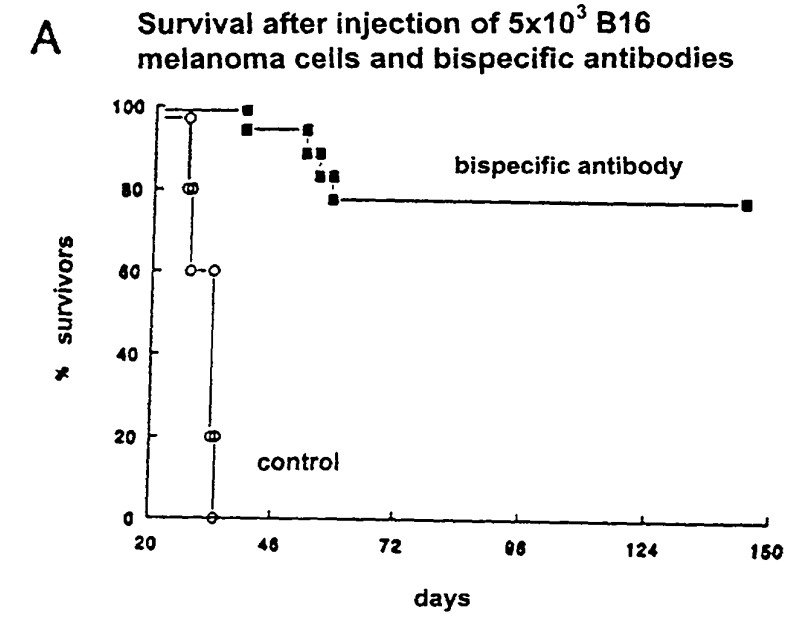


Figure 4

In vivo effectivity



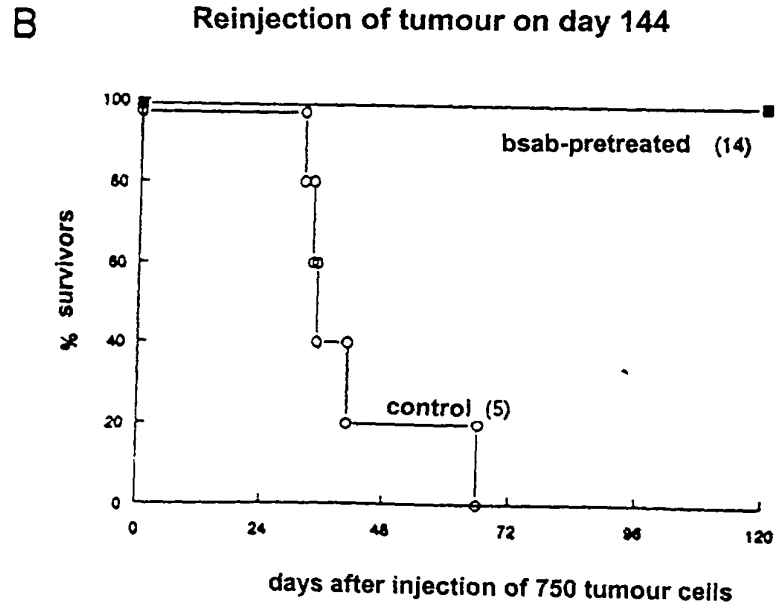


Figure 5

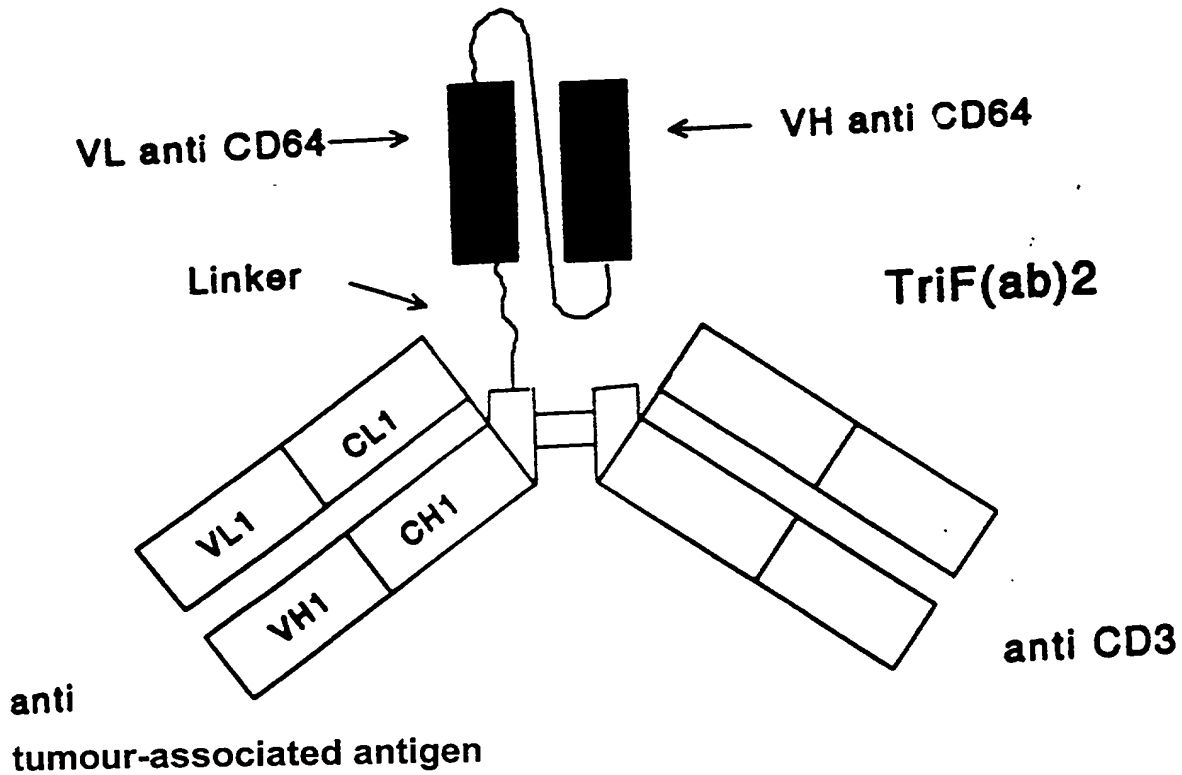
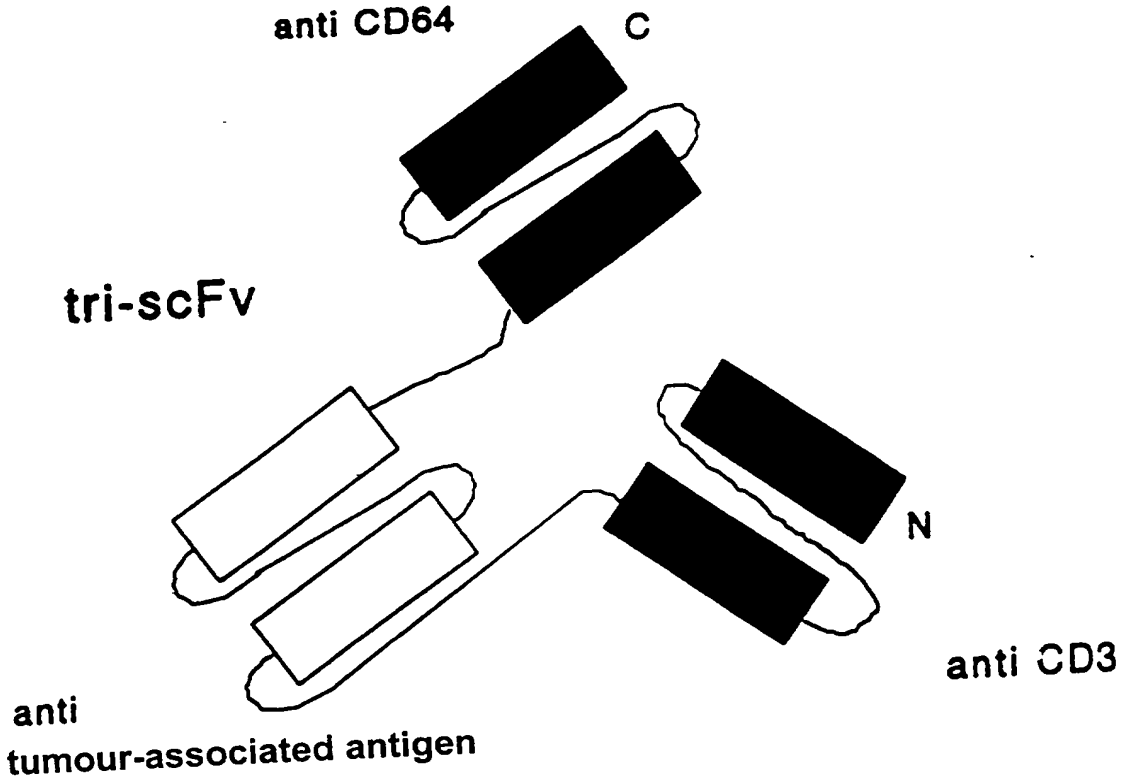

VL anti CD64
VH anti CD64
Linker
TriF(ab)2
CL1
VL1
CH1
VH1
anti CD3
anti
tumour-associated antigen

Figure 6


anti CD64
C
tri-scFv
N
anti CD3
anti
tumour-associated antigen

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I declare that:
My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **METHOD FOR EX VIVO IMMUNIZATION USING HETEROLOGOUS INTACT BISPECIFIC AND/OR TRISPECIFIC ANTIBODIES**

the specification of which _x_ is attached hereto or ____ was filed on _____ as Application No. _____ _____ and was amended on _____ (if applicable).

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56. I claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign applications(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Country	Application No.	Date of Filing	Priority Claimed Under 35 USC 119
Germany	197 25 586.8	June 17, 1997	Yes _x_ No ___
			Yes ___ No ___

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application No.	Filing Date

I claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Date of Filing	Status
		___ Patented ___ Pending ___ Abandoned
		___ Patented ___ Pending ___ Abandoned

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

M. Henry Heines, Reg. No. **28,219**
J. Georg Seka, Reg. No. **24,491**

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 1 **Horst Lindhofer**	Signature of Inventor 2 **Hans-Joachim Kolb**	Signature of Inventor 3 **Reinhard Zeidler**
Date	Date	Date
Signature of Inventor 4 **Georg Bornkamm**	Signature of Inventor 5	Signature of Inventor 6
Date	Date	Date